logists on the ABA-100, we found that about 13% of the samples (22 of 169) gave results which differed by more than 10 mg/liter.

This prompted us to test the assumption concerning the effectiveness of the blanking wavelength. The test wavelength is 650 nm and the blank wavelength is 550 nm for the phosphorus procedure on the ABA-100. We selected two patients' samples that had given discrepant results and one that had not, and diluted them 26-fold with physiological saline. These solutions were scanned (vs. saline) from 700 to 400 nm with a ratio-recording spectrophotometer (DK-2A; Beckman Instruments, Fullerton, Calif. 92634). Table 1 shows the absorbance differences for these samples. The patients' diagnoses were, respectively, cervical carcinoma, gunshot wound, and leukemia. All of these samples absorbed more at the blank wavelength than at the test wavelength and the absorbance difference varied with the patient. Results for phosphorus assays performed on these samples are also shown in the Table. These three examples serve to warn that extreme caution may have to be exercised whenever a bichromatic analyzer is used for endpoint measurements. It may not be readily apparent how much comparison work is needed before a new procedure can be used with confidence. Rather, the amount of comparison work appears to be a function of the source(s) of samples that are being assayed by the particular laboratory; that is, of whether the patient population is relatively normal or is a combination of patients who are relatively normal and those who are receiving tertiary care.

Another point also worth considering is that most of the method interferences documented to date are for procedures in which the blank and test mixtures are read at the same wavelength, information that may not be directly transferable to a bichromatic procedure.

Table 1. Absorbance Differences and Results for Phosphorus Obtained for Three Selected Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>A_{100} - A_{550}</th>
<th>Auto-Analyzer</th>
<th>ABA-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-0.004</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Heparinized plasma</td>
<td>-0.091</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Serum</td>
<td>-0.013</td>
<td>29</td>
<td>0</td>
</tr>
</tbody>
</table>

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"Albumin-Titratable Bilirubin" as an Index to Bilirubin Binding

To the Editor:

Mccluskey et al. (1) reported a modification of the Roth fluorometric method for the determination of bilirubin (2). Krasner et al. (3, 4) had previously reported their use of the method to directly titrate neonatal serum with bilirubin. The present authors emphasized that the physiological significance of "albumin-titratable bilirubin" was unknown. However, they suggested that it represented that fraction of the total bilirubin that was not tightly bound to albumin—i.e., that it represented the fraction bound to the lower-affinity site of albumin and to other components of blood (5, 6). The authors proposed that clinical management could be based on this "albumin-titratable bilirubin": "... infants with albumin-titratable bilirubin >50 mg/liter should be considered for exchange transfusion."

I believe there is adequate data in the literature to show that the Roth fluorometric method does not measure the binding of bilirubin to the high-affinity site of albumin. Saturation of the site is believed to increase greatly the risk of kernicterus. Since the proposed method does not provide a measure of saturation of that site, it should not be utilized in the clinical management of hyperbilirubinemia.

That the high-affinity site on albumin has the capacity to bind an equimolar concentration of bilirubin has been established by the peroxidase method (5), by circular dichroism (7), and by quenching of ultraviolet fluorescence (6). However, the fluorescence yield of a bilirubin/albumin mixture is linear through a bilirubin/albumin molar ratio of at least 2.0 (3, 6), implying that such fluorescence does not reflect binding to the high-affinity site.

In his original report Roth emphasized that the fluorescence remained unchanged even when the bilirubin had been photooxidized (2). He concluded that photooxidation products were equally as reactive as native bilirubin. This might be advantageous in the clinical determination of total bilirubin, but again leads to the conclusion that the method does not measure the binding of bilirubin to the high-affinity site of albumin.

Further, Krasner et al. (3, 4) examined the effect of added drugs on the fluorescence in the Roth method. Sulfisoxazole had been shown to increase the risk of kernicterus in premature infants (8). Brodersen utilized the peroxidase method to directly demonstrate the displacement of bilirubin by sulfisoxazole in vitro (9). If the Roth method were dependent on binding at the high-affinity site, one should have seen a similar effect when sulfisoxazole was added. No effect could be demonstrated, even at rather high concentrations of drug (3, 4).

The authors emphasized the correlation between "albumin-titratable bilirubin" and the bilirubin/albumin ratio as evidence that their method measured a clinically significant entity. However, such a correlation would be of clinical significance only if it resulted from a dependence of bilirubin fluorescence on binding to the high-affinity site. The evidence cited above shows that this is not the case.

The observed correlation could be the result of other physical interactions. Bilirubin fluorescence appears dependent on a hydrophobic environment (10). It may be that the albumin-mediated enhancement of fluorescence results from orientation in such an environment (10, 11).

In any event, the method does not measure bilirubin binding to the high-affinity site. It should therefore not be utilized in the clinical management of hyperbilirubinemia in the newborn.

References

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The authors of the paper in question respond (Mrs. McCluskey is deceased):

To the Editor:
Dr. Levine has correctly drawn attention to potential theoretical weaknesses in the technique developed in our laboratories. We believe, however, that his criticisms somewhat misrepresent our findings and may not be completely warranted. The following points might be made:

(a) There appears to be some confusion in the basis for Dr. Levine's critique. He equates Roth's (1) method, as used by us, with that of Krasner et al. (2, 3). Though both are fluorescent techniques, with similar (but not identical) spectral characteristics, there are substantive differences. Krasner et al. did experiments under approximately physiological conditions while our experiments were in the presence of high concentrations of phosphoric acid. Under the latter conditions, it is almost certain that the properties of the binding site have altered significantly. Thus, we do not see how it is possible for Levine to extrapolate from results reported by Krasner et al. concerning both the fluorescence yield and the results of competition experiments (with particular respect to sulfisoxazole) in order to repudiate our results.

(b) Our method also differs slightly from that of Roth in that we used human serum albumin rather than bovine serum albumin and there are distinctive differences in the mode of binding of bilirubin to these two protein molecules (4). Further, a titration of a constant concentration of bilirubin with variable albumin by our technique gives a curve very similar to the curve in Figure 2A of Beaven et al. (5), viz. a sharp inflexion at 1:1, followed by a much more gradual rise in fluorescence intensity.

(c) We would emphasize that Levine's criticisms of our work are based on results reported from experiments designed to determine the reserve binding capacity of albumin. We have felt it more important to try and assess the excess bilirubin beyond that tightly bound to albumin, as this is the problem in the jaundiced neonate.

(d) We do not believe that any meaningful statement can be made about the properties of photooxidation products of bilirubin (which could remain bound to albumin) in the absence of knowledge of their identity.

(e) The correlation between "albumin-titratable bilirubin" and the bilirubin/albumin ratio was only considered as supportive evidence that a clinically significant value was being observed. The more important correlation was considered to be with the saturation index of Odell et al. (6). This appears to measure a similar biological function by an independent method and has proved of predictive value (7).

(f) Finally, we consider it unfortunate that Dr. Levine feels that our results should be dismissed on theoretical grounds, without advancing supportive experimental evidence for his views. We have advanced the method as a possible clinical test, and follow-up studies of the patients involved is the only way of establishing its usefulness.

References

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Misuse of Calibration
Sera in Automated Laboratory Testing Instruments

To the Editor:
A common practice in the operation of automated biochemical testing instruments is to test a single sample of "calibration serum" after every 10th or 20th patients' sample. Results for each such control are used immediately to "adjust" (recalibrate) the instrument for the constituent involved. Unless the constituent can be tested with perfect precision (zero variance of the results), this practice is certain to increase the testing error, just the opposite of what is intended.

The truth of this statement is illustrated with a hypothetical example. For instance, consider glucose assay. (In the following discussion, all values are in mg/100 ml.)

Assume that an instrument was initially calibrated perfectly, and then samples from a serum pool with a "true concentration" of 100 were tested many times without adjusting the instrument. A distribution of the results like that shown at the top of Figure 1 might be obtained. Their average would be 100, but the results would vary. This variation is the "in-determinate error" (random variation) of the biochemical method, instrument, and variation of the aliquots. The vertical lines identify the ±2 SD (95%) range of the results. Each test is independent of all others, i.e., there is no "drift" or correlation of any kind among results.

In the above example, if the instrument were calibrated accurately, and left alone, it would produce the most accurate and precise results possible. But consider what happens if small, immediate adjustments are made with results of tests of single calibration samples interspersed among the patients' samples.

When the first calibration sample came along, suppose a reading of 97 was observed, and the analyst adjusted the scale to obtain 100 (first adjustment). This has the effect of adding 3 to all following results, as is illustrated. When the second calibration